Exhbt 2

LABORATORY TECHNIQUES IN BIOCHEMISTRY AND MOLECULAR BIOLOGY

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AN INTRODUCTION TO AFFINITY CHROMATOGRAPHY

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Applications of affinity chromatography to the purification of regulatory macromolecules and complex biological structures

A number of macromolecules associated with the recognition, transport, information transfer and regulatory aspects of cellular function exhibit high affinity and specificity for their complementary ligands. However, their presence in trace amounts makes their purification by conventional procedures almost impossible. Thus, selective adsorbents with biological specificity are ideally suited to the resolution and isolation of these macromolecules because they display specific binding functions with a high degree of affinity.

6.1. Antigens and antibodies

Immunoaffinity chromatography exploits the unique specificity and high affinity of antibodies for their respective antigens. In principle, the purification of antibodies from serum may be effected by binding to a column of their respective immobilised antigens. Inert proteins are washed through and the specific antibody released under conditions that disrupt the immune complex. Conversely, immobilised antibodies may be used to isolate their complementary antigens. These and a number of other applications are reviewed by Silman and Katchalski (1966), Robbins and Schneerson (1974) and Ruoslahti (1976).

Many procedures are available for insolubilising proteins to support matrices for the purification of monospecific antisera. Typically, the protein antigen is coupled to CNBr-activated agarose under mildly acid to weakly alkaline conditions to ensure retention of antibody activity. At more alkaline pH values excessive reaction of

surface lysyl residues causes distortion of the immobilised protein and reduces the antigenic and antibody activity. Furthermore, over-immobilised antigen can also fail to release the complementary antibodies because the necessary conformational change cannot occur. Sepharose 4B is activated with cyanogen bromide (33 mg/ml) at pH 10.8–11.0, thoroughly washed and added to a solution of the protein antigen. The protein (5–10 mg/ml) in a suitable buffer (pH 6–8), such as 0.1 M sodium citrate (pH 6.0) or 0.1 M sodium bicarbonate (pH 8.0), is incubated with the CNBr-activated agarose at 4°C for 18 h and then thoroughly washed to remove unbound protein. Under these conditions, coupling efficiencies $\geq 90\%$ are often observed. The washed immunoadsorbent may be stored as a suspension in neutral buffer at 4°C without loss of activity for at least 4–6 months.

The immunoadsorbent is poured into a column, equilibrated with a suitable buffer and antiserum applied. The unbound protein is washed through with starting buffer until the absorbance at 280 nm is negligible whence elution may commence. Elution of specific antibody from the immunoadsorbent may be effected, in principle at least, by establishing conditions that promote disruption of the immune complex. For example, extremes of pH and the supplementation of column irrigants with high concentrations of chaotropic ions, such as perchlorate, iodide and thiocyanate (Fig. 8.3), or protein denaturants, such as urea and guanidine hydrochloride, have proved effective. Table 6.1 lists some typical eluant conditions that have been employed in immunoaffinity chromatography. Unfortunately, the establishment of successful chromatographic conditions is still largely an empirical process. By trial and error, one can establish elution conditions which commence with the minimum and approach the maximum tolerated disruption conditions. Clearly, the most desirable system for elution is that which is the least drastic means of releasing the maximal amount of active antibody. Elution with 0.1 M glycine–HCl buffer (pH 2.5) is highly reproducible and will therefore be described in more detail. The eluant buffer is applied to the top of the column and elution continued until protein is no longer detectable in the effluent fractions. The low pH of this buffer

TABLE 6.1
Typical eluants for immunoaffinity chromatography.

Acids
0.01–1 M HCl
0.1–1 M acetic acid (pH 2.0)
1 M propionic acid
20% formic acid
0.1 M glycine–HCl (pH 2.2–2.8)
0.015 M acetic acid/0.15 M NaCl
0.1 M glycine–H_2SO_4 (pH 2.8)
1% NaCl/HCl (pH 2.0)

Bases
0.01 M NH_4OH
1 M NH_4OH/0.3 M KCl
0.05 M NH_4OH/20% glycerol (v/v)/1% BSA
0.2 M NH_4OH/0.3 M KCl/1% BSA (pH 10.8)
0.3 M 1,4-diaminobutane

Salts
3 M potassium thiocyanate
5 M KI
2.5 M NaI (pH 7.5)
2.8 M $MgCl_2$

Protein denaturants
4–8 M urea
6 M guanidine–HCl (pH 3.1)

will eventually denature the antibody, so the eluted fractions must be titrated to pH 8–8.5 with solid Tris. A second batch of antibody may be recovered by elution with 0.1 M glycine–HCl buffer (pH 2.5) containing 10% (v/v) dioxane and is neutralised in the same way. The neutralised samples are concentrated by vacuum dialysis or by dialysis against sucrose or polyethylene glycol. Under these conditions, at least 90% of the recovered protein should be antibody with demonstrable antigen-binding activity.

Immunoadsorbents based on matrices other than agarose, such as

glass, Sephadex, cellulose, metal oxides and synthetic polymers and involving a variety of chemical coupling methods are reviewed by Robbins and Schneerson (1974) and by Line and Becker (1975). Interestingly, cellulose-based antigens appear to exhibit less non-specific interaction with some proteins than the corresponding derivatised agaroses. Protein antigens may be coupled to bromo-acetyl- or carboxymethyl-cellulose and the resultant immunoadsorbents used for the purification of complementary antibodies. A typical procedure involving the use of bromoacetyl–cellulose (BAC) derivatives is as follows (Robbins and Schneerson, 1974). Cellulose is washed with acetone and anhydrous dioxane and dried over P_2O_5 to constant weight. To 10 g of the cellulose is added bromoacetic acid (100 g) in 30 ml anhydrous dioxane and the mixture stirred for 20 h at room temperature in a tightly stoppered flask. Bromoacetyl bromide (75 ml) is added and stirring continued for a further 20 h at room temperature. The HBr produced in the reaction is removed with a NaOH trap. The final solution is carefully poured into 6 litres of well stirred ice-cold water and the precipitated cellulose washed exhaustively with 0.1 M $NaHCO_3$ and water. The BAC can be stored at 4°C in the moist state prior to coupling to the protein antigens.

As a preliminary to covalent coupling of protein antigens, it is necessary to determine the optimal pH for physical adsorption to the BAC. For this, 10 mg of moist BAC is added to 10 ml antigen solution (1.0 mg/ml) in 0.15 M citrate–phosphate buffer (pH 2–7), homogenised for 5 min and the unbound protein determined. The antigen (300–500 mg) is subsequently coupled to 1 g BAC by gentle agitation for 24 h at room temperature in the citrate–phosphate buffer of optimal pH. The suspension is centrifuged and suspended in 30 ml 0.1 M $NaHCO_3$ (pH 8.9) for 24 h with gentle agitation. The antigen–cellulose is then exhaustively washed with 0.1 M $NaHCO_3$ (pH 8.9) containing 50 mM 2-aminoethanol to block residual activated groups, 8 M urea to remove non-covalently attached antigen and finally with saline. The immunoadsorbent may be stored at 4°C in saline – 0.01% sodium azide (pH 7.4).

As an alternative to immunoadsorbents prepared by covalently

coupling the antigen to an insoluble support matrix, adsorbents comprising cross-linked protein antigens offer some advantages. This method of insolubilisation is facile and the product is stable; it is frequently used for the preparation of serum proteins present in low concentrations. Typically, IgG (150 mg) is dissolved in 0.1 M potassium phosphate buffer (pH 7.2) and 0.1 ml ethyl chloroformate added with gentle stirring (Avrameas and Ternyck, 1967). After about 1 h the turbid suspension is centrifuged and the precipitate washed with water, homogenised with an all glass homogeniser and washed with 1 M glycine–HCl (pH 2.4) until protein no longer leaches into the supernatant. Appropriate volumes of antiserum are incubated for 2 h at room temperature with protein immuno-adsorbent in neutral buffer and the precipitate centrifuged and washed. The washed adsorbent is suspended in 0.1 M glycine (pH 2.2), 5.0 M KI or any of the eluants listed in Table 6.1 for 2 h at room temperature. The adsorbent is removed by centrifugation and the eluate passed through a 0.4 µm Millipore filter and equilibrated with neutral buffer. The yield of active antibody is often high (30–60%).

Immunoadsorbents have also been effective in the purification of antihapten antibodies especially when the hapten is attached to the matrix backbone by a 'spacer molecule'. It is usually possible to dissociate the hapten–antibody complex at pH 7 with a 0.1–0.5 M solution of the appropriate hapten.

6.2. Binding and transport proteins

Binding proteins are characterised by a high affinity for their complementary vitamin or hormone with dissociation constants in the range 10^{-7}–10^{-16} M and by their presence in trace amounts. For example, 1000 litres of human plasma contain only 20 mg of the vitamin B_{12} binding protein, transcobalamin II. Affinity chromatography is thus an attractive method in these cases since purification to this extent is beyond the scope of classical techniques.

The hormone or vitamin is coupled to agarose via an intermediate

'spacer' by standard organic chemistry. Thus pre-assembled ligand-spacer arms may be coupled directly to CNBr-activated agarose, or a suitable ligand analogue attached to a preformed matrix-spacer arm for assembly. For example, corticosteroid-binding globulin (CBG) may be extracted from serum by an adsorbent prepared by coupling cortisol hemisuccinate to 3,3'-diaminodipropylamino–agarose with dicyclohexylcarbodiimide in dioxane (§ 3.4.1.1) (Rosner and Bradlow, 1971). Similarly, oestradiol-binding proteins may be effectively bound by an adsorbent comprising 3-O-succinyl-[^{3}H]-oestradiol coupled to 2-aminoethyl-agarose with a carbodiimide promoted reaction. Alternatively, 17-β-oestradiol hemisuccinate may be coupled to agarose derivatives containing diaminodipropylamine, serum albumin, poly-L-lysine or poly(L-lysyl)-alanine as spacer molecules. The major problem, however, associated with affinity adsorbents for steroid binding proteins is leakage of the free steroid from the matrix. Steroids and other aromatic compounds are strongly adsorbed to some solid supports and require exhaustive washing with organic solvents for prolonged periods of time to ensure complete removal of unbound steroid. If these precautions are not taken, the free steroid binds tightly to the binding protein and inhibits the binding of the radio-labelled hormone used in the assay. This disappearance of binding capacity has often erroneously been interpreted in terms of binding to the affinity adsorbent. These problems may be circumvented by using a stable linkage between the matrix and spacer–ligand assembly and exhaustive washing conditions to remove unbound ligand.

The second major problem with the purification of binding proteins relates to their elution from the affinity adsorbent. Adsorption may be effected under batchwise conditions. For example, corticosteroid binding globulin may be adsorbed by adding cortisol-agarose batchwise to a 2-litre serum sample, gently stirring for 5 h at 4°C and thoroughly washing off unbound protein on a sintered funnel. Batchwise adsorption obviates the need for passing large volumes of serum through a relatively small column of adsorbent. The elution regime is to some extent dictated by the affinity of the ligand for